Rec'd PCT/PTO 09 APR 01

09/807254

WO 00/21364

PCT/US99/23399

A NATURAL AND SAFE ALTERNATIVE TO FUNGICIDES, BACTERIOCIDES, NEMATICIDES AND INSECTICIDES FOR PLANT PROTECTION AND AGAINST HOUSEHOLD PESTS.

This Application claims the benefit of priority to U.S. Provisional Application Serial No. 60/103,805 filed October 9, 1998.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to natural and safe compounds that are useful as insecticides, bacteriocides, fungicides and nematicides.

2. Background of the Related Art

Herbs and spices have played an important role in ancient life, as we have learned from hieroglyphics on the walls of pyramids and the Scriptures of the Bible. Spices were ranked with precious stones in the inventory of royal possessions and have played an important role in ancient medicine (J. S. Pruthi, 1980; Deans 1991).

While plants and plant extracts have long been used as medicaments, plants are also known to produce compounds which have the effect of repelling or killing insects, nematodes, bacteria and fungi that are harmful to the plants. The elaboration of natural products with deterrent effects is known for many varieties of plants.

Essential oils (etheric or volatile oils) are extracted from plant species by various extraction techniques, including steam distillation including the plants belonging to *Labiatae* and *Umbellifera*. Local populations in the Taurus Mountains in Turkey, where most of the plant species belonging to the Labiatae Family are found growing as weeds, have traditionally used these plants in teas or in oil form for thousands of years. Archaeological studies indicate that

native plant species have been a part o[illegible] for thousands of years. The teas are drunk from childhood to adulthood, and [illegible] them have a reputation for vigorous health and longevity. These plants have be[illegible]h ailments as stomach pains and intestinal infections. However, no experiments hav[illegible] to determine the *in vivo* activity of these plants against plant pathogens and househol[illegible]

The activity of essential oils has been investigated in various scientific s[illegible]search on the *in vitro* activity of essential oils against plant diseases caused by fungi, bacteria, [illegible]s and nematodes and against insects have been initiated (Singh *et al.*, 1983; Yegen,1984; Yegen *et al.* 1992; Muller-Riebau *et al.*, 1995, 1997; Saraç and Tunç 1995a; 1995b; Qasem and Abu-Blan, 1996; El-Gengaighi *et al.*, 1996; Lee *et al.*, 1997; Tunç and Sahinkaya, 1998). The works are limited to the proof of *in vitro* effects and no tests to describe practical uses were conducted.

For example, *in vitro* inhibition of growth of different phytopathogenic fungi has been described using essential oils from *Seseli indicum* (Chaturvedi and Tripathi, 1989), *Bifora radians* (Yegen, 1984), *Hyptis suaveolens* (Pandey et al. 1982), *Mentha piperita*, *Mentha citrata* and *Cymbopogon pendulus* (Matti et al. 1985), *Achryanthus aspera* (Chakravarty and Pariya, 1977), and *Peperomia pellucida* (Singh et al. 1983). There are also a few reports describing *in vitro* anti-fungal activity of wild plant species widely present as weeds in Turkey. Akgul and Kivanc (1989) have described *in vitro* activity of various Turkish spice extracts against food-borne fungi including *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp. and *Mucor* spp. Different plant extracts as well as their components had differential *in vitro* activity against various organisms i.e. *Aspergillus* spp. (Moleyar and Narasimham, 1986; Asthana et al., 1986; Farag et al., 1989b).

In vitro antifungal and antibacterial activities of aqueous extracts from *Thymbra spicata, Mentha spicata, Satureja thymbra* and *Laurus nobilis* were investigated in Petri dishes using standard assays by Akgul and Kivanc (1988a,b). The aqueous extracts of *T. spicata* and *S. thymbra* were found to have the highest anti-bacterial activity. Our own *in vitro* assays have confirmed the differential anti-fungal activity of extracts obtained from six selected plant species: *T. spicata, Satureja thymbra, L. nobilis, M. spicata, Salvia fruticosa* and *Inula viscosa,* against four plant-pathogenic fungi (MIC between 400-800 mg/mL medium) (Yegen et al., 1992). The volatile phase of these extracts was also found to be active. The activity of essential oils against *Phytophthora capsici* was better than the activities of the fungicides carbendazim and pentachlornitrobenzol. Fungitoxic components of the extracts were determined via thin layer chromatography to include carvacrol and thymol.

In vitro activity of the essential oils of *Satureja*-types against yeast have been reported (Conner and Beuchat, 1984a,b). While in our tests, essential oil extracts from *S. thymbra* and *T. spicata* had the highest activity against the growth of test fungi, Akgul and Kivanc (1989) observed a negligible anti-microbial activity against food-borne fungi *Aspergillus* sp,. *Mucor* sp., *Penicillium chrysogenum* and *Rhizopus* sp. using the essential oil from *Satureja hortensis* in comparison with oils from other spices. These studies indicate that the activity of essential oils is affected by the species of plant. Maiti *et al.* (1985) have observed that the essential oils from *M. spicata* and extracts of mentha-types have activity against 3 phytopathogens: *Rynchosporium oryzae, Drechsbra* spp. as well as *Xanthomonas campestris.* The anti-microbial activity of essential oils of other spices (i.e. sage rosemary, caraway, cumin, clove and thyme) against bacteria and fungi has been described (Farag et al. 1989a,b; Akgul and Kivanc, 1989) indicating broad range of activity among the spices. Also, extracts from leaves of the laurel tree were found

to be active against fungi *in vitro* in our tests (Yegen et al. 1992) as well as others (Akgul and Kivanc, 1989).

Singh *et al.* (1983), Asthana *et al.* (1986), Thompson and Cannon (1986), Farag *et al.* (1989), Chaturvedi and Tripathi (1989), Tripathi *et al.*, 1986; as well as Kivanc and Akgul (1990) determined minimum inhibitory concentrations of different essential oil extract from various plant species against many strains of bacteria, yeast and fungi being between 250 and 2000 ppm. The minimum inhibitory concentrations of a few individual volatile chemicals that are recommended for use in the storage of wheat grains from fungal deterioration have been determined to be 100 ppm or more *in vitro*, and most compounds were found to be phytotoxic at their minimum inhibitory concentrations (Ghosh and Nandi, 1989; Moleyar and Narasimham, 1986). Moreover, Moleyar and Narasimham (1987) described that the activity was higher at low microbial concentrations in shake cultures and the activity was decreased due to rapid detoxification of components of some essential plant oils, like menthol and citrus, by *Aspergillus niger* and *Rhizopus stolonifer* during the culturing period.

The activity of the essential oils against *Phytophthora capsici* was higher than both the fungicidal compounds, carbondazim and pentachlorintrbenzal (Yegen et al. 1992). In addition to this study, Asthana et al. (1986) found that the essential oil extracts from *Ocinum adscendens,* with minimum inhibitory concentrations of 300 μg/g, had greater growth retardation activity against *Aspergillus flavus* when compared to a few fungicides (moderately inhibitory concentrations were between 2,000 and 4,500 μg/g). While the activity of essential oils from the plants belonging to *Labiatae* and *Umbelliferae* have been studied by various scientists, practical uses in agriculture or against household pests have not yet been either studied or described.

The use of pesticides as fumigants for plant protection has become a major problem in agriculture due to soil and ground water contamination, the damaging effects of these chemicals on the ozone layer covering the Earth, and negative effects on human and animal health. Indeed, many commonly used pesticides, i.e. the ones containing carbamizadine, have been found to be carcinogenic and/or teratogenic while others, i.e methyl bromide, have been banned from use due to their negative effects on ozone layer.. Discovering naturally derived alternatives to petrochemically-derived pesticides has become essential to achieve sustainable crop production using current agricultural resources and production methods.

Alternative pesticides used for the preservation of food during transportation and storage have been sought after due to the adverse effect of these chemicals on the environment and animal health. Natural products of plants are known to be active against pathogenic organisms. For instance, plant secondary metabolites have been shown to be active *in vitro* against food poisoning bacteria (Dabbah et al. 1970; Beuchat 1976; Huthanen 1980; Tharib et al. 1983; Aktug and Karapinar 1986; Deans and Richie 1987; Deans and Svoboda 1988, 1989). The antimicrobial activity of essential oils obtained from some of Turkey's wild growing plants, as well as other weed species, plants or trees from around the world, are already known. Numerous works have dealt with the usage of essential oils to kill microorganisms causing spoilage and in the conservation of food supplies (Conner and Beuchat 1984a, 1984b; Benjilal *et. al.* 1984; De Boer *et al.* 1985; Thompson and Cannon, 1986; Moleyar and Narasimhan, 1986, 1987; Thomson, 1986; Thompson *et al.,* 1987; Akgul and Kivanc, 1988a, 1989a, 1989b; Farag *et al.* 1989a, 1989b). At the same time research regarding the use of essential oils for post-harvest prevention of the establishment of aflatoxin through *Aspergillus spp.* has advanced (Charkavarty

and Parya, 1977; Pandey *et al.*, 1982; Ghosh and Nandi, 1982; Maiti *et al.,* 1985; Tripathi *et al.*, 1986; Asthana *et al.*, 1986; Charturvedi and Tripathi, 1989).

While Thompson and Cannon (1986) observed inhibition of fungal growth using essential oils from *Mentha* sp., *Salvia* sp. and *Laurus* sp., Thompson (1986) failed to detect activity against the germination of spores from fungi belonging to genus *Aspergillus, Mucor and Rhizopus*.

The partly contrary results found in the literature, concerning the antimicrobial effect of these essential oils, led us to believe that the activity of essential oils can vary drastically due to differences not only in the plant species of origin, but between varieties and individual plants, different growing conditions, extraction procedures, as well as due to variations in strains of test micro-organisms. Therefore, we have concentrated our efforts on a few species belonging to Turkish natural fauna, doing extensive selection and breeding, and determining the best growing conditions and times for oil extraction experiments, which were not conducted in previous studies. It was found that the minimum inhibitory concentration of the volatile phase of essential oils, as well as in formulations described herein vary between about 50-200 ppm, and have the highest biological activity of any essential oil extracts described in the literature.

Several other scientists mentioned the need for discovery of a technique for practical application of essential oils in agriculture (Calderone and Spivak, 1995; Mansore et. Al. 1986; Shimoni et al, 1993). For the first time, we have demonstrated that the essential oils extracted from the plants indicated above, when emulsified in water, are not toxic at concentrations up to about 1000 ppm to many plant species, including rose, which is considered to be one of the more susceptible plants to toxic agents.

SUMMARY OF THE INVENTION

The results of our studies clearly demonstrate tat the essential oils derived from wild-growing Turkish plants can be used to combat a variety of phytopathogens, including ones which are difficult to effectively control with existing pesticides, i.e. *Phytophthora* sp. Essential oils derived from *S. thymbra* and *T. spicata* lines selected and bred for high carvacrol and thymol content are especially effective. The essential oils extracts can be used against plant foliar diseases caused by a broad spectrum of fungi and bacteria, as well as insects, when applied in varying concentrations in vapor form, or sprayed as part of an aqueous emulsion in water as well as in other preparations indicated herein. The extracts can be applied to soil as a methyl bromide replacement to kill nematodes, insects, pathogenic fungi and bacteria either alone in drip-watering systems, or together with solarization when applied in vapor form and/or by pouring or spraying to the developing root system around the growing area of plants in formulations indicated herein. In addition to their nematocidal, fungicidal, bactericidal and insecticidal activity, these extracts have beneficial side effects: they can increase the concentration of beneficial microorganisms in soils, such as fluorescent pseudomonads. The extracts also have growth promotion effects on plants such as an increased germination rate, most probably due to an increase in photosynthesis associated with increased chlorophyll content. Treated plants are greener and clearly dark green in color compared to light green to yellowish color in untreated plants when the extracts are used as a foliar spray and/or in soil applications.

The extracts have high thermostability, high volatile activity, a broad spectrum of activity against fungi, bacteria, viruses, nematodes and insects can serve as an effective replacement for the fumigant methyl bromide (which is being phased out due to its effects on stratospheric ozone, beginning in 2000) and other, hazardous chemical pesticides in agriculture.

Pinpinella anisum contains over 80% anethole. We have found that *trans*-anethole has fumigant activity that may match or exceed methyl bromide.

It is also possible to combine the essential oils with at least one other pesticide or control agents for other organisms as a part of an integrated pest management (IPM) strategy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the activity of essential oil extracts from *Thymbra spicata* (100-400 ppm) against *Phytophthora capsici* delivered via injection with zoospores (left), or naturally infected (no injections) from field soil (right).

Figure 2 shows the activity of 100-1600 ppm essential oils (a mixture of *Thymbra spicata* (30%), *Satureja thymbra* (20%) and *Origanum* spp. (40%), *Pinpinella anisum* (5%) and *Foeniculum vulgare* (5%) extracts) against *Phytophthora capsici* zoospore inoculation (1 x 10^5 zoospores/mL).

Figure 3 shows the activity of 100-1600 ppm essential oils (a mixture of *Thymbra spicata* (50%), *Satureja thymbra* (30%) and *Origanum* spp. (20%) extracts) against *Phytophthora capsici* zoospore inoculation (1 x 10^5 zoospores/mL).

Figure 4 shows the activity of 100-400 ppm essential oils (a mixture of *Thymbra spicata* (70%), *Satureja thymbra* (20%) and *Anis anisum* (10%) extracts) against *Phytophthora capsici* zoospore inoculation (1 x 10^5 zoospores/mL).

Figure 5 shows the activity of 100-400 ppm essential oils (a mixture of *Thymbra spicata* (60%), *Satureja thymbra* (20%) *Pinpinella anisum* (10%) and *Foeniculum vulgare* (10%) extracts) or 200 ppm Dazomet in soil naturally infested with nematodes, fungi and bacteria, including *Melodoigyne* spp. and *Phytophthora capsici*, in pots.

Figure 6 shows the activity of 100-400 ppm of essential oils (a mixture of *Thymbra spicata* (60%), *Satureja thymbra* (20%) *Pinpinella anisum* (10%) and *Foeniculum vulgare* (10%) extracts) or 400 ppm Dazomet in soil naturally infested with nematodes, fungi and bacteria, including *Melodoigyne* spp. and *Phytophthora capsici*, in pots.

Figure 7 shows the activity of 100-400 ppm essential oils (a mixture of *Thymbra spicata* (60%), *Satureja thymbra* (20%) *Pinpinella anisum* (10%) and *Foeniculum vulgare* (10%) extracts) or 400 ppm Dazomet in field plots naturally infested with nematodes, fungi and bacteria, including *Melodoigyne* spp. and *Phytophthora capsici*.

Figure 8 shows the activity of a bacterium (*Pseudomonas fluorescens* TR97) isolated from soils treated with essential oils, against *Phytophthora capsici* zoospore inoculation (1×10^5 zoospores/mL). All seeds were treated with a mixture of chitosan (80%) and essential oil from *Thymbra spicata* var. *spicata* (20%) and planted in sterile soil. The bacterial treatment contained 1×10^6 CFU/mL *P. fluorescens* TR97 suspended in the chitosan/essential oil mixture.

Figure 9 shows the activity of a bacterium (*Pseudomonas fluorescens* TR97) isolated from soils treated with essential oils, against *Phytophthora capsici* zoospore inoculation (1×10^5 zoospores/mL). All seeds were treated with a mixture of chitosan (80%) and essential oil from *Thymbra spicata* var. *spicata* (20%) and planted in sterile soil. The bacterial treatment contained 1×10^6 CFU/mL *P. fluorescens* TR97 suspended in the chitosan/essential oil mixture. After seeding, 100-400 ppm of essential oils embedded in perlite were added to the pots, which were covered with plastic film until germination (ca. 1 week).

Figure 10 shows activity of the volatile phase of essential oils extracted from *Thymbra spicata* against *Erwinia amylovora* on Miller-Schroth (MS) media. Droplets containing essential oils were applied to the lids of the glass petri dishes. 1, Control; 2, 50 μl/L essential oil; 3,

40μl/L essential oil; 4, 90 μl/L essential oil; 5, 60 μl/L essential oil; 6, 30 μl/L essential oil; 7, 80 μl/L essential oil; 8, 70 μl/L essential oil; 9, 20 μl/L essential oil.

Figure 11 shows the activity of the volatile phase of essential oils extracted from *Thymbra spicata* against *Erwinia amylovora* on Miller-Schroth (MS) media. The indicated amount of *Thymbra spicata* (as shown in the Figure as "thyme oil") was mixed with 0.1 mL olive oil, and droplets of the mixture were applied to the lids of the glass petri dishes.

Figure 12 shows *in vivo* activity of 200 ppm essential oil from *Thymbra spicata* or copper sulfate (commercial preparation) against *Erwinia amylovora* on pear shoots. The essential oil was applied as an aqueous emulsion to the leaves, and the shoots were inoculated with a suspension of 1 x 10^4 CFU/mL *E. amylovora*. The picture was taken seven days after inoculation. CuS indicates copper sulfate (commercial preparation).

Figure 13 shows the number of shoots exhibiting symptoms of fire blight disease in field trials on two pear varieties (SM, Santa Maria; W, Williams), 3 and 6 weeks after an application of essential oil (200 ppm in an aqueous emulsion) from *Thymbra spicata*, or copper sulfate ($CuSO_4$) (commercial preparation).

Figure 14 shows the systemic activity of essential oils on *Xanthomonas campestris* pv. *campestris* in cabbage. The soil was treated with 100-200 ppm essential oil in an aqueous emulsion from *Thymbra spicata* var. *spicata* and plants were sprayed inoculated with *X. c. campestris* (1 x 10^5 CFU/mL). Control treatments were sprayed with 100 ppm Tween 20 (emulsifying agent) in water.

Figure 15 shows the contact activities of essential oil from *Thymbra spicata* emulsified in water (100 ppm) upon foliar spray (left) or soil drench (right) applications as compared to a pathogen control treatment (center). Plants were spray inoculated with *Xanthomonas campestris*

pv. *campestris* (1×10^5 CFU/mL). The control treatment was sprayed with 100 ppm Tween 20 (emulsifying agent) in water.

Figure 16 shows the contact and systemic activities of essential oil from *Thymbra spicata* emulsified in water (100-250 ppm) upon foliar spray (left) or soil drench (right) applications. Plants were spray inoculated with *Xanthomonas campestris* pv. *campestris* (1×10^5 CFU/mL). Treating soil with the emulsion resulted in better protection from *X. c. campestris*, indicating a systemic activity.

Figure 17 shows the effect of essential oil from *Thymbra spicata* emulsified in water (100-500 ppm) on the carmine spider mite, *Tetranychus cinnabarinus*. Spider mites present on nearby infested plants were allowed to naturally infest these plants. Soil was drenched with an aqueous emulsion of essential oil in concentrations indicated (control plants were treated with water). After two weeks, the plants treated with essential oil were sprayed with a 200 ppm aqueous emulsion of the same essential oil. After foliar treatment, no spider mites were detected on any of the sprayed plants. The inset illustrates the difference in leaf color between the control (leftmost) treatment and the essential oil treatments (the latter are a darker green).

Figure 18 shows the toxicity of anethole vapors to the adults of *T. confusum* and *S. oryzae* and the larvae of *E. kuehniella*.

Figure 19 shows the toxicity of anethole vapors to the eggs of *T. confusum* and *E. kuehniella*.

DETAILED DESCRIPTION OF THE INVENTION

The essentials oils covered in this application may be extracted from plant species belonging *Labiateae* and *Umbelliferae*. Suitable plants may include specimens in the genera

Thymbra, Satureja, Origanum, Corydothymus, Pinpinella and *Foeniculum*. Examples of plant species in the Family *Labiatae* include *Thymbra spicata* var. *spicata* (L), *Satureja thymbra* (L), *Origanum majorana* (L), *Corydothymus capitatus* (L.) Reichb. fil., *Origanum vulgare* (L) subsp. *hirtum* (Link.) Let., *Origanum solymicum* P.H. Davis, *Origanum spyleum* (L), *Origanum bilgeri* P.H. Davis, *Origanum minutiflorum* O. Schwarts & P.H. Davis,*Organum saccatum* P.H. Davis, *Origanum sriacum* var. *bevanii* (Holmes) Letswart, *Origanum onites* (L), and *Origanum majorana* (L). Examples of plant species in the Family *Umbellifera* include *Pinpinella anisum* L. and *Foeniculum vulgare* Miller. These plant species were identified by Prof. Dr. Huseyin Sumbul, Akdeniz University according to Volumes 4 and 7 of the series of books written by L.H. Davis, entitled: *Flora of Turkey and Eastern Aegean Islands*. The plant species identified herein are illustrative only and are not intended to be limiting. Other plant species containing any one of the components of the essential oil extracts, as identified by gas chromatography (conducted in Gottingen University, Germany, Akdeniz University, and METU, Turkey using methods described by Shultze et al. 1986) are encompassed within the scope of the invention.

The components of essential oil extracts were verified by chemical analysis (Akgul, 1986; Arrebola et al. 1994; Capone et al. 1988; Muller-Riebau et al. 1995; Philianos et al. 1984; Ravid and Putievski 1984, 1986; Shukla and Tripathi, 1991; Sarer et al. 1985; Schaffer et al. 1986). The compounds identified from extracts of these plant species (in alphabetical order) are: cis-anethole, trans-anethole, anisaldehyde, anis ketone, anisole,β-bisabolene, borneol, bornyl acetate, cadinene, camphene, camphor, Δ-3-carene, Δ-4-carene, carophyllene, carvone, carvacrol, γ-caryophyllene, cinnamic aldehyde, citral, citronellal, cineol, 1,8-cineole, *p*-cymene, *p*-cymene-8-ol, decanal, estragole, eugenol, eugenyl acetate, α-fenchene, fenchole, fenchone, geranial, geraniol, geranyl acetate, isoborneol, lavanduol, limonene, linalool, linalyl acetate, menthol,

menthone, menthyl acetate, *cis*-p-menth-2-en-1-ol, *trans*-p-menth-2-en-1-ol, methoxy phenyl acetone, methyl chavicol, methyleugenol, methylinone, 2-methylpentan-3-one, myrcene, nerol, nonanal, *cis*-β-ocimene, *trans*-β-ocimene, octanal, 3-octanol, α-pinene, β-pinene, α-phelladrene, β-phelladrene, pulegone, sabinene, *cis*-sabinene hydrate, *trans*-sabinene hydrate, γ-terminene, terpenyl acetate, α-terpinene, γ-terpinene, terpinene-4-ol, a-terpineol, β-terpineol, terpinolene, 2,3,5,6-tetramethylphenol, α-thujene, thymil acetate, thymol, and tricyclene.

Although all of these components have some biological activity, they work synergistically to provide a broad spectrum of activity. Total essential oil extracts, as extracted from plants, have as good or often better activity than individual synthesized components, indicating that the components of essential oil extracts from these plant species act synergistically. Therefore, the extracts can act as multisite pesticides, and their activity is relatively stable.

The major active components are: carvacrol, thymol, cymene and anethole. A suitable product for commercial use may contain various individual plant extracts or combinations of individual plant extracts. For Example: *T. spicata* (10-90%), *S. thymbra* (10-90 %) and *Origanum spp.* (5-30 %) can be mixed to provide optimal activity against bacteria, viruses and fungi, and *T. spicata* (10-90 %), *S. thymbra* (10-75), *P. anisum* (0.5-90 %) and *F. vulgare* (0.5-90 %) can be mixed to provide optimal activity against insects and nematodes. The activity of each extract or combination of extracts against detrimental organisms varies. That is, a mixture containing more carvacrol and thymol is more active against microbial pathogens, whereas a mixture containing more carvacrol and anethole is more effective against insects and nematodes.

The invention also comprises chemically synthesized essential oil components which can be used as pesticides and for pharmaceuticals. Anethole may be synthesized relatively easily, for example, and is a effective pesticide suitably used in the invention to replace methyl bromide for

fumigation, in storage applications and the like. Combinations of synthetic essential oil components may also be produced and used within the scope of the invention.

The LD 50 values of the components of the essential oils (see Duke et al. 1992) indicate that they are not highly toxic or teratogenic to humans and animals. Although concentrated oils are toxic to plants, and may cause a temporary painful inflammation on human skin, so far no toxic effects to plants and/or to the environment have been recognized when the oils are used in appropriate concentrations and in the preparations described herein.

Plants with Enhanced Essential Oil Activity

Carvacrol, thymol and anethole content was increased by up to ten-fold of the original, wild-grown plants in four selected plant species, using various selection and conventional breeding techniques. Currently, the seedlings of obtained cuttings for *Labiatae spp.* or from seeds of *Umbelliformae spp.* from the selected lines are growing under greenhouse conditions for distribution to farmers, in order to prevent possible disturbances to the natural ecology of the mountains, which could be caused by the overharvesting of wild plants. None of the new varieties of these species have been previously grown in the United States. The plant lines selected for enriched essential oil content are *Thymbra spicata* var. *spicata* (L) Line Ant97- 364-48, *Satureja thymbra* (L) Line Ant98-28-103, *Pinpinella anisum* (L) Line Ant98-223-137, and *Foeniculum vulgare* (L) Line Ant98-89-62.

It was also determined that the highest essential oil content occurs during the flowering period, which is late June to early July in the Mediterranean region of Turkey, for the two perennial *Labiatae* species. Both *Umbellifera* species are annual plants and since seeds are used for extraction of essential oils, seeding period is the best time for essential oil collection.

It is also possible to develop transgenic plants that have enhanced production of essential oils or essential oil components. In particular, plants selected for enhanced production of *trans*-anethole, carbacrol, and/or thymol would be expected to have more potent essential oil than normal plants of the same species.

The essential oil extracts can be used against plant diseases caused by a broad spectrum of fungi, bacteria and nematodes as well as insects, when applied in varying concentrations mixed with other oils, in vapor form, or sprayed as part of an aqueous emulsion in water as well as in other preparations indicated below. The extracts can be applied to soil when embedded in perlite, in granular form, powder form, or emulsified in water and applied via drip-watering systems. The extracts can also be used together with solarization when applied in vapor or any other form as well as by pouring or spraying aqueous emulsions around the growing area of plants in formulations indicated below. In addition to their nematicidal, fungicidal, bactericidal and insecticidal activity, these extracts have beneficial side effects: when used as a soil and/or foliar spray and/or soil application, they can increase the concentration of beneficial microorganisms in soils, such as fluorescent pseudomonads (*Pseudomonas fluorescens*), and have growth promotion effects on plants (i.e. increased germination rate, foliage production and height). The growth promotion effects are most probably due to an increase in photosynthesis associated with increased chlorophyll content (treated plants are greener and clearly dark green in color compared to light green to yellowish color on untreated plants), or modifications in other plant metabolic systems.

Essential oils as extracted are not soluble in water and are phytotoxic in undiluted oil form, and therefore, cannot be used for direct foliar applications, and can be either adsorbed by soil particles or toxic to plant roots when directly applied to soil. Therefore, the oils have to be

formulated in granules or powders or absorbed in a carrier, such as perlite or vermiculite, for soil applications and have to be emulsified in water for soil and foliar applications. Formulations may be those found as commercial formulations used for other pesticides.

For soil applications, the essential oil may be mixed with a carrier substance, such as a porous substance. Suitable porous carrier substances include perlite and vermiculite. For these applications, at least about 0.5g of essential oil is combined with about 10-50g of the carrier substance, such as perlite, vermiculite or mixtures thereof. The applications using the carrier substances may be applied to soil alone, or in combination with solarization. Solarization may be conducted by covering the treated soil area with a transparent, impervious covering such as polyethylene, and the soil kept moist for up to about 6 weeks. Plants can be transplanted into the soil immediately or several days after application of the essential oil treatment.

For application to irrigation water, essential oil concentrations may be about 10-1000 ppm. More preferably, the concentration of essential oil is about 100-1000 ppm.

For fogging applications, essential oils in emulsion form or diluted in other oils, or full concentrate are atomized and applied over plants and/or soil in a storage area or greenhouse, for example. Typically a concentration of about 100-1000 ppm in air of the storage area is suitably used.

The extracts obtained from the plants indicated above are also active, and being considered non-toxic they can be used safely against, household insects, including, but not limited to mites, spiders, dust mites, house flies, cockroaches, mosquitoes, fruit and garbage flies, ticks and other pests, when applied in vapor or aerosol form, in dust or granular formulations, diluted in carrier oils, extracted with solvents which dissolve essential oils or sprayed as aqueous emulsions or in any other form onto places where insects are present, or on body parts and

clothing. Essential oil extracts also can be used, when applied as a vapor or in other forms, to control stored product insects (storage pests) to replace methyl bromide and/or other insecticides when applied by atomizers, as vapors, i.e., from heated extracts or mixed in paint.

They can also be used as mixed with liquid paraffin as in any form indicated above for protecting citrus and other fruits during transportation and storage, since coating orange and grapefruit fruits with paraffin containing about 0.5-90% essential oils increased storage time up to ten fold. Although, increased concentrations of essential oils may result in an unpalatable odor, this can be eliminated by adjusting the oil content and mixture according to needs of commercial applications.

The results of current work clearly demonstrate that the essential oil of *T. spicata* has a practical use as a soil fungicide and can be used as such, since good results against *Phythophthora capsici* in greenhouse tests and in two field tests were achieved. In these early tests, pure essential oil was adsorbed in perlite, which protected the essential oil from adsorption by other soil components, protected the plants against the phytotoxic effects of the undiluted oil and ensured an equal distribution of the oil on the ground (Yegen et al, 1998). Although this application of the essential oils proved to be effective, is was further determined that applications of the extract in granular or dust formulations, or application as an aqueous emulsion directly to irrigation water, in combination with covering the soil for a period of time prior to transplanting, are more effective and practical methods. The tests where an aqueous emulsion of the essential oils poured on the soil around the plants (via irrigation or pouring by hand), or directly sprayed on plants, provided protection against fungal, bacterial, viral pathogens, against nematodes and insects on or around plants. We have further developed and tested practical uses against household insects and other pests.

Soil chemical fumigants and other treatments do not specifically target particular pests or pathogens. Therefore, they have negative effects on whole soil microfloral and microfaunal communities, including plant- and soil-beneficial organisms. The essential oils described in this application, however, appear to work selectively. Detrimental organisms are targeted while populations of soil- and plant-beneficial microflora, including fluorescent pseudomonads and actinomycetes, are not decreased. In fact, population densities of these organisms actually increase after treatment, most likely due to the reduction in competition from phytopathogens and probable increases in available nutrients from lysed phytopathogen cells. These beneficial soil organisms may influence plant disease resistance and growth either directly, or indirectly through natural, microbially-mediated changes to the soil environment. A fluorescent pseudomonad (*Pseudomonas fluorescens* TR97) able to degrade these essential oils has been isolated, indicating that these oils can be metabolized. Unlike organochlorines and other recalcitrant pesticidal compounds, components of the essential oils will not accumulate in soils or in living tissues. Some of these isolates also appear to act indirectly (as they have little direct antimicrobial activity) to inhibit the growth of soil phytopathogens, either through the induction of plant defense responses, or through suppression of the pathogens via competition.

The invention will now be described in further detail by reference to Examples, which are intended to be illustrative of the invention, and not limiting. The scope of the invention is defined in the appended claims.

EXAMPLES

1. Materials and Methods

Distillation of essential oils: 500 mL of distilled, deionized water were combined with 32 g of dried plant material or seed derived from one or several of the species indicated above

and distilled with a Clevenger apparatus until 400 mL distillate was obtained. Extracted twice with petroleum ether, the ether phase was separated and dried in a rotary evaporator at 45°C to obtain the maximum amount of essential oils. Essential oils can easily be separated from water using a separatory funnel. Under commercial factory conditions, about 1 kg of dried plant material or seed is generally mixed with about 10 liter of water or extracted in plant material with vapor in commercial continuous extraction systems. It is not necessary to use petroleum ether unless all the oil content is needed. Water and plant content may vary according to distillation technique.

Preparation of essential oils for practical use in plant protection: Essential oils from the plants were emulsified in water. For a 1000 ppm emulsion, about 1 mL essential oil containing extract(s) from one or several plant species was mixed with various concentrations of Tween 20 or other commercial detergents (the optimal concentration is about 1 mL essential oil extract(s) dissolved in about 1 mL Tween 20) and added to about 1 L water. For optimal emulsification, the water is acidic. Therefore, about 1 drop of concentrated hydrochloric acid per L of water was used to bring down the pH of the water to approximately 5.0.

Activity of Essential Oils Against *Phytophthora capsici* Under Greenhouse and Field Conditions:

(a) Soil tests: Soil samples were obtained from infected fields in Kumluca, Antalya, Turkey, placed in the plastic pots and inoculated in the growth-chambers. Field tests were performed at the same site where soil was obtained. 2 kg field soil was sifted through a 2 mm sifter to a plastic container and the soil dampened with sterile distilled water to 75 % saturation. Essential oils extracted from *T. spicata* and various mixtures of extracts were added in to the soil

in an aqueous emulsion form by adding the emulsion to the irrigation water. Plots were covered with polyethylene plastic film to prevent evaporation of the essential oils. The activity of the essential oils was compared to 400 mg/kg Dazomet (Basamid 980 active ingredient).

(b) Activity Against Pepper Root Rot Disease Caused by *P. capsici:* 500 g of sifted soil was placed into a plastic container and dampened with distilled water to 75% to saturation. Each container was inoculated with 2 mL zoospore suspension (1100 zoospores/mL) of *P. capsici*. After 1 week 50, 100, and 200 mg essential oils of from *T. spicata,* or various mixtures of essential oils as indicated above, formulated into aqueous emulsions, were mixed into the soil. The resulting concentrations of essential oil concentrations in the soil was 100, 200, 400 and/or 1,600 mg/kg (See Figure Legends). The containers were covered with airtight plastic film and incubated at 25°C for 5 days in a climate-controlled room. The film was then removed, and after 3 days of aeration, 15 pepper (*Capsicum annum)* seeds from a variety susceptible to *P. capsici* (Demre, Vegetable seed Co. Antalya, Turkey) were seeded into each pot. The number of living plants was determined two weeks after plantation.

Field tests were conducted at a site located near Kumluca, Antalya, Turkey. Essential oil extracts were added in water as indicated above. Parcels were covered for 5 days with plastic film. The seeds from a susceptible pepper variety of *C. annuum* (Demre) were seeded in high density equally in each plot, 8 days after the fumigation treatment. The number of diseased plants as well as the dry weight of the plants (dried at 105 C for 24 hr), per m^2, was determined. The differential weight as well as the total sum of the weight of the plants were determined.

ACTIVITY OF ESSENTIAL OIL EXTRACT FROM *THYMBRA SPICATA* L. var. *SPICATA* ON VARIOUS BACTERIA

(a) Isolation of the Essential Oil

Top leaves and flowers of *Thymbra spicata*, *Satureja thymbra*, and *Origanum* spp. were collected from the wild at the time of flowering, while seeds were collected from *Pinpinella anisum* and *Foeniculum vulgare*. 32 g of dried plant material or seed was steam-distilled with 500 mL of distilled water until 400 mL of condensed liquid was obtained. The separation from the water was conducted twice by 800 mL of petroleum ether. The extract was steam-distilled at 45°C until the petroleum ether (boiling range 60-80°C) was completely evaporated. The essential oil was stored in the dark at 4°C until further analysis.

(b) Contact and Volatile Phase Effects of the Essential Oil

Essential oil obtained from *Thymbra spicata* L. var. *spicata* was assessed for its contact and volatile phase effects towards several economically important plant pathogens: *Agrobacterium tumefaciens, Clavibacter michiganensis* subsp. *michiganensis, Erwinia amylovora, Erwinia carotovora* pv. *carotovora, Pseudomonas syringae* pv. *syringae* and *Xanthomonas axonopodis* pv. *vesicatoria.* Bacteria (1 x 10^5 CFU/mL) were incubated at 25°C in nutrient broth (NB) containing 20-1,280 μg/mL essential oil. The minimum bacterial concentration (the lowest concentration yielding fewer than 0.1% survivors) was determined by plating 0.1 mL of the flask contents onto nutrient agar (NA) plates. The bacterial colonies on the NA plates were counted at 24 hr intervals for three days. Control flasks contained NB and 1 x 10^5 CFU/mL of test bacteria.

Glass petri dishes of 100 mL capacity were used in the determination of volatile phase effects of essential oil. The test bacteria (1 x 10^5 CFU/mL) were plated onto NA, and the plates were dried under aseptic conditions under a laminar flow hood. Different concentrations of essential oil (doses of 2-128 μl, corresponding to 20 to 1280 μg/mL air) were applied to the lids of the petri dishes. The bottom of the petri dish was immediately placed on the lid and sealed

by parafilm to prevent diffusion of essential oil from the dish. The sealed, inverted petri dishes were incubated at 25°C for 3 days. The seals were removed after 3 days to release the volatile essential oil. The petri dishes were incubated for an additional 3 days before determining the minimum inhibitory concentration (MIC) of oil by counting bacterial colonies. The MSTAT statistical program was used to compare treatments via Duncan`s multiple range test (p=0.05). The MIC was determined based on the equation of the regression analysis (Dimond et al., 1941).

Examples 1-3: Use of Essential Oils in Plant Protection:

1. Foliar Applications:

(a) Spray Applications:

Essential oils extracted from plants can be emulsified in water and sprayed onto plants at weekly intervals, or as needed, similarly to conventional pesticides. They can be used in concentrations ranging from about 1 to about 1000 ppm according to the pest or pathogen targeted and/or to the sensitivity of the plant species (the best concentration is about 200 ppm for most organisms and plant species). Essential oils also appear to improve plant health in the absence of disease.

(b) Fogging with Atomizers:

Essential oils can be used in vapor form, mixed with other oils, dissolved in solvents which dissolve essential oils, or in emulsion form to fog greenhouses or other buildings to kill pathogens and pests using ultra low volume nozzles: about 1-3 L per 1000 m^2 of greenhouse area, down to about 0.25 ppm per 1000 m^2. Concentrations can be increased for application against house pests and/or to fog greenhouses.

Example 2. Soil Applications:

(a) Soil tests from the Field: Essential oil concentrations obtained from sampled field soil and from the 2 kg field sifted field soil were between 100 to 1600 mg/kg soil in growth chamber studies or 100-400 mg/kg soil in field tests. The application of essential oils even in the lowest concentration (100 mg/kg) reduced the numbers of total microorganisms 7-25% even 1 day after application, compared to the controls. Populations of all three microorganisms (fungi, bacteria and actinomycetes) were significantly reduced when applied at 400 mg/kg in comparison to the control (approximately 40% for the fungi, 70% for the bacteria and 40% for actinomycetes). The populations of fungi and bacteria recovered more quickly than the actinomycetes.

(b) Activity of Essential Oils Against Pepper Root Rot Disease: The result of growth chamber tests were shown in the Fig. 1, 2, 3, 4, 5 and 6. The number of infected plants upon pouring various essential oil emulsions were significantly lower compared to controls. The fresh weight per plant and fresh weight per pot were increased compared to controls upon treatment with essential oils (data not shown).

The total number of healthy and infected plants in the field experiments is summarized in Fig. 7. Treatment of the soil with the aqueous solutions of essential oil extracts significantly increased the total number of plants per m^2. Essential oil extracts provided better control compared to Dazomet.

(c) Application as Imbedded in Perlite or Vermiculite:

Essential oils extracted from the plant species indicated above, alone or in combination, can be embedded into a carrier such as perlite or vermiculite as extracted in powder or granular formulations as formulated in commercial preparations, diluted in other oils, dissolved in

solvents which dissolve essential oils or in an aqueous emulsified form. A minimum of 0.5 g of essential oils were mixed with 10-50 g of perlite or vermiculite. The perlite or vermiculite was then sprinkled on the soil surface or mixed to a 5-10 cm depth into soil, using a commercial fertilizer applicator, to cover an area of one (1) square meter. The surface of the soil was then covered with a plastic sheet for at least 2 days for vaporization. Other commercially available chemicals, such as materials known to induce systemic disease resistance in plants, including chitin and chitosan, biological control agents able to survive exposure to the oils or components of biological control agents can be added to the same materials. Applications of perlite or vermiculite can be used alone or in combination with solarization. For solarization, the soil surface should be covered with polyethylene and kept moist for up to six weeks. Plants can be planted or transplanted into the soil immediately or several days after application according to the plant species used.

(d) Application into irrigation water:

Essential oils diluted in other oils, dissolved in solvents which dissolve essential oils, or used in an emulsified form in water as described above, can be added in a manner similar to fertilizers to irrigation water (at about 10-1000 ppm concentrations, optimal concentrations generally range from about 100-200 ppm), and also directly to plants in drip-water irrigated greenhouses to reduce, minimize or completely halt the diseases caused by soil pathogenic fungi, bacteria, or nematodes and the damage caused by insects. First irrigation can be made before transplanting, and the soil can be covered with polyethylene to increase the vapor effect and to allow for the integrated use of solarization. The application into irrigation water can be continued during the growing season to increase activity.

Example 3. Storage Applications:

The essential oils extracted from the plants indicated above, and the vapors of these oils, can be used to kill storage pests and pathogens. Fogging, as indicated above, increases the activity of the oils due to a higher distribution rate to a larger area. Essential oils may also be used when applied as vapors, i.e., from heated extracts or mixed in paint (preferably, an oil-based paint). Heating is not required for vaporization, however, heating improves vaporization. Bombs can be made by formulating the essential oils in preparations similar to preparations used for methyl bromide in which the essential oil compositions are packaged in pressurized cans. Low concentrations (about 25-1000 ppm in air volume of storage area) of vapors from essential oils can provide a good alternative to methyl bromide to kill insects or microorganisms attacking produce under storage and transportation conditions. Notably, anethole is highly effective for this application and may be extracted from plants or is easily synthesized. We have found that although both *cis*- and *trans*-anethole are effective, *trans*-anethole is more effective. Essential oils can also be used as mixed with liquid paraffin as in any form indicated above for protecting citrus and other fruits during transportation and storage.

Example 4: ACTIVITY OF ESSENTIAL OIL EXTRACT FROM *THYMBRA SPICATA* L. var. *SPICATA* ON VARIOUS BACTERIA

(a) Contact and volatile phase effect of Essential Oil

The contact and volatile phase effect of different concentrations of essential oil differed against the various plant pathogenic bacteria tested. The number of the living bacterial cells decreased as the dose of the essential oil increased (Tables 1-3). The volatile phase of the essential oil was more effective on *E. amylovora, X. a. vesicatoria, C. m. michiganensis* and *A. tumefaciens* than on *P s. syringae* and *E. carotovora* (Table 2, 3).

Table 1. The activity f the Volatile Phase of Essential Oils from *Thymbra spicata* against *Erwinia amylovora*. A droplet containing essential oil was applied to the lid of the petri dish in the essential oil treatments.

Essential Oil (*T. spicata*) droplet (µl/L)	**Experiment I* Number of colonies ****	**Experiment II Number of colonies**
20	211.7 b	203.3 b
30	206.3 b	196.3 b
40	202.7 b	194.0 b
50	149.0 c	140.3 c
60	147.7 c	135.0 c
70	129.3 cd	126.7 cd
80	126.0 cd	120.7 cd
90	101.3 d	93.0 d
100	0.0 e	0.0 e
200	0.0 e	0.0 e
300	0.0 e	0.0 e
400	0.0 e	0.0 e
500	0.0 e	0.0 e
STREPTOMYCIN (50 µg/mL, in media)	0.0 e	0.0 e
CONTROL	256.7 a	271.0 a

* No significant differences between treatments in Experiments I and II were detected using t-tests ($p=0.05$).

** Differences between treatments were identified using Duncan's analysis ($p=0.05$). Treatments followed by the same letter are not significantly different.

Table 2. Contact Effect of Essential Oil of *Thymbra spicata spicata* on Plant Pathogenic Bacteria

	TEST BACTERIA* (CFU/mL)					
Dose μg/mL	At.	C.m.m.	E.c.c.	E.a.	P.s.s.	X.a.v.
0	1.71×10^8 a	1.05×10^8 a	3.60×10^8 a	1.89×10^8 a	6.05×10^8 a	4.73×10^8 a
20	1.49×10^8 a	8.90×10^7 a	2.25×10^8 b	1.20×10^8 b	2.36×10^8 b	4.29×10^8 a
40	1.21×10^7 a	7.20×10^7 a	1.66×10^8 c	8.33×10^7 c	2.12×10^8 b	2.73×10^8 b
80	8.60×10^7 ab	6.30×10^7 ab	1.22×10^8 ed	4.33×10^7 d	4.40×10^7 c	2.17×10^5 bc
160	8.40×10^7 ab	5.80×10^7 ab	9.60×10^7 de	1.83×10^7 e	4.00×10^7 c	1.50×10^4 c
320	6.34×10^3 bc	1.80×10^7 bc	5.00×10^7 ef	0.00 f	3.30×10^7 c	7.70×10^2 d
640	0.00 c	0.00 c	0.00 f	0.00 f	1.10×10^7 d	0.00 e
1280	0.00 c	0.00 c	0.00 f	0.00 f	0.00 c	0.00 e

* Values are means of four replicates. Values within one column followed by different letters are significantly different at $p = 0.05$ (Duncan's Multiple Range Test). A.t. *Agrobacterium tumefaciens*; C.m.m. *Clavibacter michiganensis*; E.c.c. *Erwinia carotovora* pv. *carotovora*; E.a. *Erwinia amylovora*; P.s.s. *Pseudomonas syringae* pv. *syringae*; X.a.v. *Xanthomonas axonopodis* pv. *vesicatoria*.

Table 3. Volatile Phase Effect of the Essential Oil of *Thymbra spicata spicata* on Plant Pathogenic Bacteria

	Test Bacteria* (CFU/petri dish)					
Dose μg/mL air	A.t.	C.m.m.	E.c.c.	E.a.	P.s.s.	X.a.v.
0	133 a	195	79 a	237 a	185 a	192 a
20	71 b	76 b	61 ab	171 b	172 a	148 ab
40	26 c	40 c	57 abc	82 c	171 a	104 b
80	17 c	11 d	52 bc	0 d	169 a	0 c
160	0 c	0 d	49 bc	0 d	137 a	0 c
320	0 c	0 d	35 c	0d	24 b	0 c
640	0 c	0 d	0 d	0 d	16 b	0 c
1280	0 c	0 d	0 d	0 d	0 b	0 c

* Values are means of four replicates. Values within one column followed by different letters are significantly different at $p = 0.05$ (Duncan's Multiple Range Test). A.t. *Agrobacterium tumefaciens*; C.m.m. *Clavibacter michiganensis* subsp. *michiganensis*; E.c.c. *Erwinia carotovora* pv. *carotovora*; E.a. *Erwinia amylovora*; P.s.s. *Pseudomonas syringae* pv. *syringae*; X.a.v. *Xanthomonas axonopodis* pv. *vesicatoria*.

The essential oil in the contact effect tests showed MIC ranging from 276 μg/mL to 413 μg/mL (Table 4). In most cases, the volatile phase effect of the essential oil was more toxic to the test bacteria than contact with the essential oil. The essential oil in the volatile phase effect tests showed an MIC ranging from 41 μg/mL to 684 μg/mL (Table 4).

Table 4. MIC of the E.O. of *T. s. spicata* to plant pathogenic bacteria

Bacteria	MIC (μg/mL) in contact effect tests	MIC (μg/mL air) in volatile phase effect tests
A. tumefaciens	328	98
C. m. michiganensis	405	91
E. c. carotovora	413	569
E. amylovora	276	59
P s. syringae	344	684
X a. vesicatoria	323	41

Example 5. Use of essential oils against household pests:

The essential oils extracted from the plants indicated above are also active against household pests, and could replace other pesticides or deterrents. These oils can be used as extracted, in vapor phase, diluted in carrier oils, dissolved in solvents which dissolve essential oils or emulsified in water to spray homes or other buildings and/or clothing to kill or deter insects (i.e. ants, house flies, spiders, mites, fleas, mosquitoes, termites, ticks, etc.). They also can be applied to skin in a cream or spray form to deter insects such as mosquitoes and ticks.

Example 6. Activity of essential oil extracts against honeybee parasites:

The activity of essential oils against chalkboard of honeybee caused by *Ascosphaera apis*

(Maasen ex Claussen) Olive & Spiltoir was determined in several honeybee colonies. Aqueous emulsions of essential oil extracts were not active against honeybees (some mortality at about 1000 ppm) although they were found to be extremely active against the parasite. Both aqueous and volatile phase of essential oils in insecticidal preparations killed the *Ascosphaera* sp. under laboratory and apiary treatments (about 100-500 ppm). In the same experiments reductions in the natural populations of the parasitic mites (*Varrora jacobsoni*) were also observed. Essential oils to treat honeybee parasites are used in the form of aqueous emulsions, diluted in other oils, in the form of dust or powders, in vapor form, or any other formulation described herein.

Example 7: Activity

The minimum inhibitory concentrations (MIC) of essential oils extracted from the plants indicated above against four fungi belonging to major plant pathogens (*Fusarium moniliforme, Rhizoctonia solani, Sclerotinia sclerotium and Phytophthora capsici*) were found to be between 300 to 800 µg/mL of the medium used (PDA). In addition, a droplet of essential oils (0.1 mL each), when applied to the lid of petri dishes (10 cm diameter) completely inhibited the growth of these fungi (indicated above), demonstrating that the active ingredient was also inhibitory in vapour form.

The essential oils of *Thymbra spicata* var. *spicata* showed the best activity against *P.*

capsici, the agent of pepper blight, both in greenhouse and in field studies. So far there is no fungicide effective against *P. capsici,* except soil sterilization with methyl bromide, and *Phytophthora* species are a major pathogen of peppers and many other crops in many areas of the world, especially in the Mediterranean region. In greenhouse trials with naturally infested soil, the number of healthy plants of *Capsicum annuum* was increased from 4 per pot to 10 per pot after treatment with different concentrations of essential oils. The number of infected plants was reduced, respectively, from 7 to 2 plants per pot. In field trials, the number of healthy plants per square meter was significantly increased in treated plots. The rate of germination of pepper seeds was 75% better than controls and 20% better than methyl bromide (statistically significant). The soil fumigant Dazomet (BASF, Basamid), claimed as the only broad spectrum pesticide alternative to methyl bromide (EPA Publication on Alternatives to Methyl Bromide, U.S. EPA), and methyl bromide were used as a positive control. Dazomet appeared to be significantly less effective than the controls as well as treatment with the essential oil, in both greenhouse and field trials. Investigations of the activity of the soil microflora showed that the essential oils had a lesser impact on beneficial soil microflora and microfauna than methyl bromide and Dazomet. The essential oils reduced the population of beneficial soil fungi and bacteria up to 40%, while the dehydrogenase activity was reduced only 10%. Dazomet, however, reduced the population of beneficial soil fungi and bacteria up to 90%, and dehydrogenase activity decreased by about

50%.

Further studies conducted under field conditions, using essential oils absorbed into perlite, or sprayed as emulsions in water indicated that the activity of these oils is retained under field conditions. 50 g perlite containing 10 mL of essential oil extracts was placed 5 cm deep in the soil at equal intervals. In addition, essential oils were also applied (200-300 ppm) emulsified in 5L water/m^2. Soil was covered with polyethylene for a period of 5 days for solarization and left open for three days prior to transplanting. Presently methyl bromide is used together with soil solarization, which takes 3-4 days or solarization is used alone, requiring for soil to be covered with polyethylene for up to six weeks, which is too long for cut-flower production. Aqueous emulsion application may be repeated every 15 days to increase the protection level once the plants are established.

The antimicrobial activity of essential oil extracted from *Thymbra spicata* var. *spicata,* and various mixtures of oils from the plant species indicated above, was also studied against bacterial plant pathogens including: *Erwinia amylovora, E. carotovora* pv. *carotovora, Clavibacter michiganensis* var. *michiganensis, Pseudomonas syringae* pv. *syringae, Agrobacterium tumefaciens* and *Xanthomonas axonopodis* pv. *vesicatoria* when applied in vapor phase or mixed in to the growing medium. Minimum inhibitory concentrations (MIC) of essential oils in media ranged from 200-400 µg/mL against all bacteria tested. MIC of the

volatile phase of essential oils were ranged from 40-650 μg/mL of air, indicating that volatile phase was more effective to all bacteria except *E. c. carotovora* and *P. s. syringae* than its contact effect.

We have also determined whether essential oils could replace methyl bromide for storage applications. Essential oils from *Thymbra spicata* var. *spicata* and the other plants indicated above, in various mixtures, were placed in a container for growing insects containing *Tribolium confusum, Stophilus oryzae* and *Ephestia kuehniella*. Essential oils killed over 95 % of the insects when used at a 100-200 μl/L air concentration within 1-6 days. In a similar study, essential oils from various plant species used in vapor phase killed 99% of spider mites (*Tetranychus cinnabarinus*) and cotton aphids (*Aphis gossypii*) within 2-3 days of exposure (0.5 μl/L) under laboratory conditions. Essential oils also had very high activity against these insects when sprayed on to leaves as emulsion in water (1-1000 μl/L, preferably 100-200 μl/L) under greenhouse and field conditions. Essential oils also have high activity in vapor form and/or sprayed as diluted in other oils, dissolved in solvents which dissolve essential oils, or as emulsions in water as pesticide and/or deterrent against mosquitoes, mites, cockroaches, flies, house flies, termites and ticks. Notably, *F. vulgare* and *P. anisum* have higher concentrations of anethole in their essential oils than *T. spicata*. Therefore, essential oils derived from *F. vulgare* and *P. anisum* are more effective, and work at lower concentrations than those derived

from *T. spicata*. Essential oils from these plants may also be used in combination.

Extracts of plant species naturally grown in Turkey are found to be potent anti-fungal, bactericidal, nematocidal and insecticidal agents. They also improve plant health and growth by improving (or avoiding damage to) indigenous beneficial microflora and microfauna. According to EPA publications, essential oils from these species are not considered toxic to animals or to the environment. Our discovery would replace traditionally used petrochemically derived pesticides and fumigants, particularly methyl bromide, which will be banned from use in the early 21st century.

Example 8: The Effect of Essential Oils from *Origanum* spp. Against *Xanthomortus axonopodis* pv. *vesicatoria*

Concentrations of etheric oils from *Origanum* spp. (*in vitro* and *in vivo*) were determined based on spectrophotometric absorbance at 600 nm (Table 6). Streptomycin sulphate added to a flask culture completely inhibited the growth of *X. a. vesicatoria* in 24 hr, and had a bactericidal effect: living bacteria were not detected at any point up to three days following the addition of the antibiotic. Essential oil also had a bactericidal effect at concentrations of 1,000 μg/mL or greater. Control suspensions reached stationary phase 24 hr after inoculation.

The essential oil also demonstrated antibacterial activity at relatively low concentrations; 250 μg/mL, after 24 hrs and 500 μg/mL, after 48 hrs. The decrease in the bacteriostatic activity of the essential oil over time is probably due to the volatilization and diffusion of active volatile

ingredients (Table 6).

Example 9: Potential Effect of the Essential Oil of Oregano *in vivo*

Doses of 100-1000 μg/mL essential oil were tested against *X. axonopodis* pv. *vesicatoria* on the leaves of pepper plants. The plants were first kept in dew chamber in 100% relative humidity for 60 hrs, in order to provide water-soaked leaves on the plants. The essential oil extracts (emulsified in water as described above, at concentrations ranging from 100-1000 μg/mL essential oil), streptomycin sulphate (200 μg/mL) and sterile tap water as a control were sprayed in 5 mL water. Plants were inoculated with a bacterial suspension (100 CFU/mL) and the percentage of water soaking was determined 7 days after inoculations. The essential oil extracts significantly reduced the occurrence of leaf spot disease caused by *X. axonopodis* pv. *vesicatoria* on pepper plants, compared to occurrence of the disease on the control plants and the plants treated with streptomycin (Table 5). The inhibition rate of the disease increased as the dose of the essential oil increased (Table 6). Streptomycin treatment completely prevented the leaf spot diseases on leaves of pepper plants. Any level of phytotoxicity of the tested concentration of the essential oil was not detected under the experimental conditions.

Table 5. The Activities of Essential Oil f Oregano on *Xanthomonas axonopodis* pv. *vesicatoria in vivo*	
Concentration of Essential Oil (μg/mL)	**Inhibition Rate (%)**
0	0
100	25
250	35
500	45
1000	60
Streptomycin (200 μg/mL)	100

The results of this study confirmed the effectiveness of the essential oil extracts against a bacterial pathogen of pepper. The antibacterial activity can further be increased by using essential oil extracts in combination with low levels of various antibiotics. Essential oils from *T. spicata* and various mixtures of essential oils had higher activity compared to *Origanum* spp. where close to 60, 70 and 90% inhibition was detected with 250, 500 and 1000 μg/mL concentrations, respectively.

Table 6. The Activities of Essential Oil of Oregano n *Xanthornonas axonopodis* pv. *vesicatoria in vitro*						
Concentration of Essential Oil (µg/mL)	Absorbance (600nm)	Inhibition Rate (%)	Absorbance (600nm)	Inhibition Rate (%)	Absorbance (600 nm)	Inhibition Rate (%)
1	0.26	59.38	0.32	50	0.5	21.88
12.5	0.19	70.31	0.28	56.25	0.35	45.31
25	0.16	75	0.25	60.94	0.3	45.31
50	0.14	78.13	0.22	65.63	0.3	53.13
100	0.11	82.81	0.22	65.63	0.25	53.13
200	0.03	95.31	0.22	65.63	0.18	60.94
250	0.00	100	0.18	71.88	0.13	71.88
500	0.00	100	0.09	85.94	0.00	79.69
1000	0.00	100	0.00	100	0.00	100
2000	0.00	100	0.00	100	0.00	100
Streptomycin	0.00	100	0.00	100	0.00	100
Control	0.64	0.00	0.64	0.00	0.64	0.00

Example 10: The Activity of Essential Oils from *Thymbra spicata* Against Fire Blight Disease caused by *Erwinia amylovora*

Fire blight disease, caused by *Erwinia amylovora*, of is one of the most damaging disease of fruit trees all around the world. *In vitro* and *in vivo* activities of essential oils from *Thymbra spicata* against *Erwinia amylovora* was determined. In order to determine *in vitro* activity of the vapor phase of the essential oils, droplets containing various concentrations of essential oil were applied to the lids of inverted petri dishes containing Miller-Schroth (MS) media, which had been previously inoculated with *E. amylovora* (Table 1, Figs. 10-11).

In vivo activity was determined in laboratory tests using apple tree shoots, where essential oil extracts were sprayed in emulsions prepared as described earlier. The shoots were then

sprayed with a bacterial solution containing 1 x 10^4 CFU/mL. There was no phytotoxicity observed with applications of up to about 1000 ppm essential oil concentration. Essential oil extracts of *Thymbra spicata* (about 200 ppm) reduced disease intensity 90% compared to controls (Fig. 12).

(a) Field Tests:

Field tests were conducted at two pear orchards near Isparta, Turkey, on two different pear varieties, disease susceptible (Santa Maria) and partially resistant (Williams). The application of essential oils from *T. spicata* (about 200 ppm.) was compared to the application of copper sulfate (at commercial rates). Weekly application of essential oil extracts in an aqueous emulsion (about 200 ppm) protected both varieties significantly against fire blight disease, and the occurrence of the disease was completely eliminated in the partial resistant variety (Fig. 13).

Example 11: Determination of Activity of Essential Oils from *Thymbra spicata* Against *Xanthomonas campestris* pv. *campestris* in Cabbage

Cabbage plants from a disease susceptible (Perfect Ball) variety were grown under growth chamber conditions (22-25°C with an 18 hr photoperiod provided by sodium lamps). Seedlings were treated with aqueous emulsions of essential oils from *Thymbra spicata* var. *spicata* at about 100, 250 and 500 ppm/plant concentrations, applied as either soil drench or foliar spray applications (Figs 14, 15 and 16) 12 hr prior to inoculations with *Xanthomonas campestris* pv. *campestris* (XCC), the causal agent of black rot disease of crucifers. The results indicated that application of an emulsion of essential oils from *T. spicata* to the soil provided better protection against this disease than foliar application. This may indicate that essential oils have systemic activity in the plants against pathogenic organisms.

Example 12: Activity of Essential Oils Against Carmine Spider Mite (*Tetranycus cinnabarinus*) in Pepper

Pepper plants were treated with 100, 200 and 500 ppm concentrations of essential oil in aqueous emulsions (prepared as indicated above) from *Thymbra spicata* var. *spicata* via initial soil drenchings, followed two weeks later by a foliar application of a 200 ppm emulsion. The soil drenchings alone reduced infestations with mites by 60% compared to untreated controls. Foliar application completely killed all spider mites within minutes after application (Fig. 17), and plants remained uninfested for up to a week. The emulsions appear to work better than any insecticide we have tested. Combinations of extracts from *Thymbra spicata* (60 %), *Satureja thymbra* (20 %), *Anis anisum* (10%) and *Foeniculum vulgare* (10%) , also killed the mites as effectively as the extract from *T. spicata* when used alone. The differences among the applications may be due to differences in systemic activity.

Emulsions of essential oils have a very high contact activity, as well as a volatile phase activity, against small insects, i.e. Drosophila, spiders, mosquitoes, sugar ants and aphids. An emulsion containing about 100-1000 ppm essential oil will kill over 50% of the sampled insects within 0.5-3 minutes after spraying.

The antifungal and antibacterial activity of the essential oils is derived from their ability to lyse the cell membranes of these organisms. Cell membrane lysis of zoospores and bacteria was observed directly via optical microscopy.

Essential oils can be used against *Phytophthora fragaria* and nematodes infesting strawberries and other crops dependent on methyl bromide fumigation. The fact that these oils are derived from a natural source instead of a petrochemical one, and have no known toxicities

when used in diluted form as described in this patent, would make treatment with these oils preferable to treatment with a chemical such as Dazomet- particularly for organic growers.

Example 13. Fumigant Activity of Anethole Against Different Stages of Three Important Stored Product Insects

We conducted a study of the fumigant activity of anethole against different stages of three important food storage insects: eggs and adults of the confused flour beetle, *Tribolium confusum*, adults of the rice weevil, *Sitophilus oryzae* (L.), and eggs and larvae of the Mediterranean flour moth, *Ephestia kuehniella* Zeller.

Materials and methods: *T. confusum* were reared on a mixture of wheat flour, bran and yeast; *E. kuehniella* were reared on ground wheat, and *S. oryzae* were reared on wheat grains. Insect rearing and all experimental procedures were carried out at 26 ± 1°C and 65 ± 5% r.h. *Trans*-anethole (Sigma) used in the tests was of 99% purity. Adults (≤ 14 days old) of *T. confusum* and *S. oryzae* and larvae (13-16 days old) of *E. kuehniella* were exposed to anethole in small nylon gauze bags containing rearing food. Twenty insects were placed in each bag to make one replicate. Three replicates for each dose and exposure time combination were taken.

Eggs (0-24 hours old) of *T. confusum* and *E. kuehniella* were exposed on cloning plates (Nunc, Denmark) modified for this purpose. (Tunç et al., 1997). A set of cloning plates consisted of a bottom plate with 60 microwells and a cover plate which had 60 holes drilled over the microwells. A seriograph cloth was placed between two plates to avoid escape of hatched larvae while allowing air circulation. One egg was accommodated in each microwell, for a total of 60 eggs per plate. Each plate was divided into three sections, each accommodating 20 eggs which formed one replicate. Three replicates were used for each concentration and exposure time

combination. All experiments on the adults, larvae and eggs were repeated twice, thus the total number of replicates for each dose x exposure time was six.

The test chambers were 650 mL glass jars with screw-top lids. Anethole, diluted in acetone was applied on a blotting paper strip which measured 3 x 8 cm. The blotting paper was attached to the lower side of the jar's lid with adhesive tape. Anethole doses of 1.88 to 15.0 mg diluted in 200 μl acetone (corresponding to 2.9 to 23.1 mg/L air for the eggs and adults of *T. confusum*, the adults of *S. oryzae* and the eggs of *E. kuehniella*) and doses of 15.0 to 120.0 mg/L air (corresponding to 23.1 to 184.8 mg/L air for the larvae of *E. kuehniella*) were applied with an automatic pipette. Only acetone was applied in control jars. Acetone was evaporated for 14 to 22 seconds before the lids were fitted to the jars. After exposure for 24, 48 or 96 hours, bags and plates containing insects were taken out of jars. Final mortality counts were taken 3 days later for adults and larvae and 11 to 9 days later for the eggs of *T. confusum* and *E. kuehniella*, respectively. Mortality data were corrected for natural mortality in controls and were subjected to probit analysis to estimate LT_{50} and LT_{99} values (Sokal and Rohlf, 1973).

Results: Vapors of anethole were found to be toxic to all test insects: the eggs and adults of *T, confusum*, the adults of *S. oryzae*, and the eggs and larvae of *E. kuehniella* (Fig. 18). However, the toxicity was variable among the species tested. Doses of 11.6, 23.1 and 184.8 mg anethole/L air were required at varying exposure periods to achieve 100% mortality in the adults of *S. oryzae* and *T. confusum*, and the larvae of *E. kuehniella*, respectively.

The time required for 99% mortality at 23.1 mg/L air was $\leq$ 24 and 35.5 hours in the adults of *S. oryzae* and *T. confusum*, respectively (Table 7). A lower dose, 11.6 mg/L air would be sufficient, however, to achieve the same mortality at a prolonged exposure time, e.g., 61.7 hours in *S. oryzae*. A much higher dose (such as 92.4 mg/L air) and a longer exposure period

(such as 89.1 hours) were required for 99% mortality in the larvae of *E. kuehniella* (Table 8).

Table 7. LT50 and LT99 values for *T. confusum* adults and eggs, *S. oryzae* adults and *E. kuehniella* eggs exposed to various doses of anethole vapors at 24-96 hours

Dose (mg/L air)	*T. confusum* (adults) LT_{50} (h)	LT_{99} (h)	*S. oryzae* (adults) LT_{50} (h)	LT_{99} (h)	*T. confusum* (eggs) LT_{50} (h)	LT_{99} (h)	*E. kuehniella* (eggs) LT_{50} (h)	LT_{99} (h)
2.9	988.6	*	229.6	*	208.9	*	40.7	*
5.8	134.9	*	29.2	*	29.8	*	2.5	*
11.6	10.8	875.0	13.5	61.7	2.8	218.8	1.1	117.5
23.1	3.4	35.5	**	**	**	**	**	**

* Estimated LT_{50} and LT_{99} values were too far beyond tested exposure range to be reliable
** It was not possible to estimate LT_{50} and LT_{99} values due to 100% mortality in all exposure periods tested.

Table 8. LT_{50} and LT_{99} values for the larvae of *E. kuehniella* exposed to various doses of anethole vapors at 24-96 hours

Dose (mg/L air)	LT_{50} (h)	LT_{99} (h)
23.1	323.6	*
46.2	22.0	*
92.4	4.3	89.1
184.8	**	**

* Estimated LT_{50} and LT_{99} values were too far beyond tested exposure range to be reliable
** It was not possible to estimate LT_{50} and LT_{99} values due to 100% mortality in all exposure periods tested.

Anethole was also toxic to the eggs of *T. confusum* and *E. kuehniella*. A concentration of 23.1 mg/L air was enough to achieve 100% mortality at ≤ 24 hours in the eggs of both species (Fig. 19). Based on the LT_{50} values, the eggs of *E. kuehniella* were more sensitive than the eggs of *T. confusum* (Table 7). These results also indicated that the eggs were more sensitive than the other stages of the species tested (e.g. the adults in *T. confusum* and the larvae of *E. kuehniella*). On the basis of the LT_{50} values, sensitivity to anethole of the species and their different stages

in descending order was *E. kuehniella* eggs, *T. confusum* eggs, *S. oryzae* adults, *T. confusum* adults, and *E. kuehniella* larvae.

The results clearly indicate that anethole possesses a significant fumigant potential against different stages of three important stored product insect species. Anethole apparently was more toxic than its parent compound, essential oil of anise, against the species tested. For instance, for a 95% mortality at 24 hours, at least 108 and 135 µl anise oil/L air (specific gravity of anise oil was approximately 1.0) was required against the adults of *S. oryzae* and *T. confusum*, respectively (Saraç and Tunç, 1995) while 23.1 mg anethole/L air was sufficient to achieve the same mortality in the adults of both species in this Example.

Ho et al. (1997) reported that anethole had fumigant activity against the adults of *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* Motschulsky. However, the way in which the data was presented does not allow any comparison of the results of the two investigations.

Our data suggests for the first time that anethole has a fumigant activity that matches or surpasses that of methyl bromide. In experiments designed as space treatments, anethole was capable of killing 100% of the eggs of *T. confusum* and *E. kuehniella* and the adults of *S. oryzae* at a dose of approximately 23.1 g/m^3 air and at $\leq$ 24 hours exposure. The doses of methyl bromide recommended for treatment of various commodities (e.g. for cereals, tobacco, and raisins and dried figs) in Turkey are 25, 35, and 40 g/m^3, respectively, at 24 hours (Anonymous, 1995).

Certain stages of some insect species may tolerate the doses of anethole that cause 100% mortality in other species, as exemplified by the larvae of *E. kuehniella* in the present Example. It was demonstrated that this could be overcome by increasing the dose.

Apart from its fumigant activity, anethole was reported to have contact toxicity against

the eggs, larvae, and adults of *T. confusum* and the adults of *S. zeamais* and exhibited a repellent effect against the adults of *T. confusum* (Ho et al., 1997). Anethole was also shown to be toxic and totally inhibit the reproductive activity of a serious fruit pest, *Ceratitus capitata* Wied.(the Mediterranean fruit fly) when orally administered (Bazzoni et al., 1997).

Example 14: Mixtures of Essential Oils from *T. spicata*, *P. anisum* and *F. vulgare* Are Effective in Killing Insects

Mixtures of essential oils were applied in various concentrations in different formulations to test efficacy in killing insects. Efficacy can be found in the range of about 50 to 1000 ppm. When essential oil is derived from *Umbelliferae* alone (such as from *P. anisum*) as little as about 1 ppm is effective in killing a variety of insects.

Tests were conducted in jars, on plants and by spraying in air. The results are summarized in Table 9.

Table 9. An Mixture of Essential Oils (80% *T. spicata*, 10% *P. anisum*, and 10% *F. vulgare)* are Effective in Killing Insects.

Insect	Dose as an emulsion in water (in ppm)	Results
Spider Mites	100	90% killed in 1 min.
Ants	100	50% killed in 3 min.; 100% killed in 10 min.
Aphids	200	100% killed in closed jars in 5 min.; 50% killed on plants in 10 min.
Mosquitoes	200 in emulsion	100% killed when used as a spray
German cockroaches	500	100% killed in 10 min.

Example 15: Essential Oil Compositions Repel Mosquitoes

A mixture of essential oils was prepared from in the following proportions: 40% *T.*

spicata, 10% *F. vulgare*, 10% *O. ssp.*, 30% *S. thymbra*. The mixture was formulated in compositions comprising 10-50% essential oils in olive oil. Application of the essential oil composition was effective in repelling mosquitoes for 3-4 hours. No bites were received in the time period tested. Olive oil controls were not effective in repelling mosquitoes. The 50% composition was the most effective, but slight burning sensation was reported. A 30% composition formulated in a cream was effective for repelling mosquitoes for 1.5 to 2 hours. An example of a cream formula composition containing the essential oils described above was formulated as shown in Table 10.

Table 10. Formulations for a 1 kg skin cream c ntaining essential oil as an insect repellent	
Ingredient	**Amount in grams**
Beeswax	75-100
Borax	10
Castor oil	0-20
Cocoa butter	0-20
Distilled water	313.5-443.5
Essential oil composition	6-200
Glycerin (vegetable)	15
Grapefruit seed extract**	0-10
Jojoba oil	35-50
Olive Oil	200-325
Sweet almond oil	50
Wheat germ oil	25
Shea butter	10-15

** This preservative may be replaced with a combination of 1 g/kg methyl paraben and 0.5g/kg propyl paraben.

REFERENCES CITED

1. Akgul, A. 1986. Studies on the essential oils from Turkish fennel seeds (*Foeniculum vulgare* M. var. Dulce). Pages 487-489. In *Progress in Essential Oil Research*. Edited by E.-J. Brunke, Walter DeGruyter & Co., New York.

2. Akgul, A. and M. Kivanc. 1988a. Inhibitory effects of six Turkish thyme-like spices on some common food-borne bacteria. Nahrung 32, 201-203.

3. Akgul, A. and M. Kivanc. 1988b. Inhibitory effects of selected Turkish spices and oregano components on some foodborne fungi. Int. J. Fd Microbiol. 6, 263-268.

4. Akgul, A. and M. Kivanc. 1989. Sensitivity of four foodborne moulds to essential oils from Turkish spices, herbs and citrus peel. J. Sci. Fd. Agric. 47, 129-132.

5. Anonymous. 1995. Manual of Pest Control. Ministry of Agriculture and Rural Affairs, Ankara (In Turkish).

6. Arrebola, M.L., M. C. Navarro, J. Jimenez and F. A. Ocana. 1994. Yield and composition of the essential oil of *Thymus serpylloides* subsp. *serpylloides*. Phytochemistry. 36, 67-72.

7. Asthana, A., N. N. Tropathi, S. N. Dixit: 1986. Fungitoxic and phytotoxic studies with essential oil of *Ocimum adscendens*. J. Phytopath. 117, 152-159.

8. Bazzoni, E., G. S. Passino, M. D. L. Moretti and R. Proto. 1997. Toxicity of anethole and its effects on egg production of *Ceratitis capitata* Wied. (Dip. Tephritidae). Ann of Appl. Biol. 131, 369-374.

9. Benjilal, B., A. T. Elakari, A. Ayadi, and M. Ihal. 1984. Method to study antimicrobial effects of essential oils: application to the antifungal activity of six Moroccan essences. J. Fd Prod. 47, 748-752.

10. Capone, W., C. Mascia, M. Melis and L. Spanneda. 1988. Determination of terpenic compounds in the essential oil from *Satureja thymbra* growing in Sardinia. J. Chromatography 457, 427-430.

11. Chakravarty, D. K. and S. N. Pariya. 1977. Inhibition of germination of phytopathogenic fungi in some Indian medicinal plant extracts. Z. PflKrankh. PflSchutz. 84, 221-223.

12. Chaturvedi, R. V., S. C. Tripathi: Fungitoxic, physico-chemical and phytotoxic properties of essential oil of *Seseli indicum* W. & A. J. Phytopath. 124, 316-322, 1989.

13. Coats, J. R. 1994. Risks from natural versus synthetic insecticides. Ann. Rev. Entomol. 39, 489-515.

14. Conner, D. E., L. R. Beuchat. 1984a. Sensitivity of heat-stressed yeasts to essential oils of plants Appl. Envir. Microbiol. 47, 229-233.

15. Conner, D. E. and L. R. Beuchat. 1984b. Effects of essential oil from plants on growth of food spoilage yeasts. J. Fd. Sci. 49, 429-434.

16. Deans, S.G. 1991. Evaluation of antimicrobial activity of essential (volatile) oils. Pages 309-320. In *Essential Oils and Waxes: Modern Methods of Plant Analysis* vol. 12. Edited by H. F. Linskens and J.F. Jackson, Springer-Verlag, New York.

17. De Boer, E., W. M. Spiegelenberg, and F. W. Janssen. 1985. Microbiology of spices and herbs. Antonie van Leeuwenhoek 51, 435-438.

18. Duke, J.A. 1992. Handbook of biologically active phytochemicals and their activities. CRC Press, Boca Raton, Florida USA.

19. El-Gengaihi, S.E., S.A.A. Amer and S.M. Mohamed. 1996. Biological activity of thyme oil against *Tetranychus urticae*. Koch. Anz. Schadlingskde. Pflanzenschutz, Umweltschutz. 69, 157-159.

20. Farag, R. S., Z. Y. Daw, F. M. Hewedi and G. S. A. El-Baroty. 1989a. Antimicrobial activity of some Egyptian spice essential oils. J. Fd. Prod. 52, 665-667.

21. Farag, R. S., Z. Y. Daw and S. H. Abo-Raya. 1989b. Influence of some spice oils on *Aspergillus parasiticus* growth and production of Aflatoxin in a synthetic medium. J. Fd. Sci. 54, 74-76.

22. Ghosh, J.,B. Nandi and N. Fries. 1982. Use of some volatile compounds in the preservation of wheat grains from fungal deterioration in storage under Indian conditions. *Z.* PflKrankh. PflSchutz 89, 410-418.

23. Ho, S. H., Y. Ma, and Y. Huang. 1997. Anethole, a potential insecticide from *Illicium verum* Hook F., against two stored product insects. Int. Pest Control 39, 50-51.

24. Karapinar, M.: The effects of citrus oils and some spices on growth and aflatoxin production by *Aspergillus parasiticus* NRRL 2999. Int. J. Fd. Microbiol. 2, 239-245, 1985.

25. Karapinar, M.: Influence of menthol and thymol on *Aspergillus parasiticus* growth amid production of aflatoxins in a synthetic medium. Mitt. Geb. Lebensmittelunters. u. Hyg. 81, 287-295, 1990.

26. Kivanc, M., A. Akgül: Mould growth on black table olives and prevention by sorbic acid, methyl-eugenol and spice essential oil. Nahrung 34 , 369-373, 1990

27. Klingauf, F., H. J. Bestman, O. Vostrovsky, and K. Michaelis. 1983. Wirkung von ätherischen ölen auf Schadinsekten. Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie 4, 123-126.

28. Lee, S.G., I.K. Soon, J.A. Young, B.K. Joon and Y.L. Boum. 1997. Effectiveness of carvacrol derived from *Thujopsis dolabrata* var. *hondai* sawdust against *Thecodiplosis japonensis* (Diptera: Cecidomyiidae) Pesticide Sci. 49, 119-124.

29. Maitti, D., C. R. Kole and C. Sen. 1985. Antimicrobial efficacy of some essential oils. – Z. PflKrankh. PflSchutz. 92, 64-68.

30. Moleyar, V., and P. Narasimham. 1986. Antifungal activity of some essential oil

components. Fd. Microbiol. 3, 331-336

31. Moleyar, V., and P. Narasimham. 1987. Detoxification of essential oil components (citral and menthol) by *Aspergillus niger and Rhizopus stolonifer*. J. Sci. Fd. Agric. 39, 239-246.

32. Muller-Riebau, F. Berger, F. and O. Yegen. 1995. Chemical composition and fungitoxic properties to phytopathogenic fungi of essential oils of selected aromatic plants growing wild in Turkey. J. of Agricultural and Food Chemistry 43, 2262-2266.

33. Pandey, D. K., N.N. Tripathi, R.D. Tripathi and S.N. Dixit. 1982 Fungitoxic and phytotoxic properties of the essential oil of *Hyptis suaveolens*. Z. PflKrankh. PflSchutz. 89, 344-349.

34. Philianos, S.M., T. Andriopoulou-Athanassoula and A. Loukis. 1984. Constituents of the essential oil of *Satureja thymbra* L., from different regions of Greece. Int. J. Crude Drug Research, 22, 145-149.

35. Pruthi, J.S. 1980. *Spices and Condiments: Chemistry, Microbiology, Technology*. Academic Press.

36. Quasem, J.R. and H.A. Abu-Blan. 1996. Fungicidal activity of some common weed extracts against different plant pathogenic fungi. J. Phytopath. 144, 157-161.

37. Ravid, U., and E. Putievsky, 1985. Composition of essential oils of *Thymbra spicata* and *Satureja thymbra*. chemotypes. Planta Med. 4, 337-338.

38. Ravid, U., and E. Putievsky, 1986. Carvacrol and thymol chemotypes of East Mediterranean wild Labiatae herbs. In *Progress in Essential Oil Research*. Brunke, F.J. (Ed.) Walter de Gruyter Berlin, 163-167.

39. Saraç, A. and I. Tunç. 1995a. Toxicity of essential oil vapours to stored-product insects. Z. PflKrankh. PflSchutz 102, 69-74.

40. Saraç, A. and I. Tunç. 1995b. Residual toxicity and repellency of essential oils to stored-product insects. Z. PflKrankh. PflSchutz 102, 429-434.

41. Sarer, E., J.J.C. Scheffer, A.M. Janssen, A. Baerheim-Svendsen. 1985. Composition of the essential oil of *Origanum majorana* grown in different localities in Turkey. Pages 209-212. In *Essential Oils and Aromatic Plants*, edited by A. Baerheim-Svendsen and J.J.C. Scheffer, Nijhoff/Junk, Dordrecht, the Netherlands.

42. Scheffer, J.J.C., A. Looman, A. Baerheim-Svendsen and E. Sarer. 1986. The essential oils of three Origanum species grown in Turkey. Pages 151-156 in *Progress in Essential Oil Research.* Brunke, F.J. (Ed.) Walter de Gruyter, Berlin.

43. Shaaya, E., U. Ravid, N. Paster, B. Juven, U. Zisman, and V. Pissarev. 1991. Fumigant toxicity of essential oils against four major stored-product insects. J. Chem. Ecol. 17, 499-504.

44. Shukla, H.S. and S.C. Tripathi. 1987. Antifungal substance in the essential oil of anise (*Pinpinella anisum* L.) Env. Biol. Chem. 51, 1991-1993.

45. Singh, D. K., R. Dikshit, S. N. Dixit: Antifungal studies of *Peperonin pellucida.* Beitr. Biol. Pfl. 58, 357-368, 1983.

46. Sokal, R. R. and F. J. Rohlf. 1973. Introduction to Biostatistics. Freeman and Company, San Fransisco, California.

47. Thompson, D. P. 1986. Effect of essential oils on spore germination of *Rhizopus, Mucor*

and *Aspergillus* species. Mycologia 78, 482-485.

48. Thompson, D. P. and C. Cannon. 1986. Toxicity of essential oils on toxigenic and nontoxigenic fungi. Bull. Envir. Contam. Toxicol. 36, 527-532.

49. Thompson, D. P., C. Cannon, G. Parter and T. Tsefamichael. 1987. Mycoassay of fluorescent fractions from seven essential oils. Bull. Environ. Contain. Toxicol. 39, 688-695.

50. Tripathi, S. C., S. P. Singh and S. Dube. 1986. Studies on antifungal properties of essential oil of *Trachyspermum ammi* (L.) Sprague. J. Phytopath. 116, 113-120.

51. Tunç, I., S. Sahinkaya. 1998. Sensitivity of two greenhouse pests to vapor of essential oils. Entomologia Experimentalis et Applicata 86, 183-187.

52. Tunç, I., F. Erler, F. Dağli and Ö Çaliş. 1997. Insecticidal activity of acetone vapours. J. Stored Prod. Res. 33, 181-185.

53. S. Sahinkaya. 1998. Sensitivity of two greenhouse pests to vapor of essential oils. Entomologia Experimentalis et Applicata 86, 183-187.

54. Yegen, O.: Kokarot *(Bifora radians)* Bunyesindeki Ucucu Yagin Fungitoksik Potansiyeli. Ankara Universitesi Ziraat Fakültesi Yayinlar 909, Bil. Ar. ve Incel. 531, 24, 1984.

55. Yegen, O., B. Berger, R. Heitefuss, 1992. Investigations on the fungitoxicity of extracts of six selected plants from Turkey against Phytopathogenic fungi. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz. Journal of Plant Diseases and Protection 99, 349-359.

56. Yegen, O., A. Unlu and B.M. Berger. 1998. Einsatz und nebenwirkungen auf

bodenmikrobielle aktivitaten des etherischen ols aus *Thymbra spicata* bei der bekamfung der wurzelhalskrankheit an paprika *Phythophthora capsici*. Z. PflKrankh. PflSchutz. 105, 602-610.